Malaysian Journal of Microbiology, Vol 12(1) 2016, pp. 102-111

Malaysian Journal of Microbiology
Published by Malaysian Society of Microbiology
(In SCOPUS since 2011)

Molecular diversity pattern of intestinal lactic acid bacteria in Cemani chicken, Indonesian native chicken, as revealed by terminal restriction fragment length polymorphisms

Siti Nur Jannah[1*], Achmad Dinoto[2], Komang Gede Wiryawan[3] and Iman Rusmana[4]

[1]Department of Biology, Diponegoro University. Jl. Prof Soedarto, Tembalang, Semarang, 50275, Central Java, Indonesia.
[2]Research Center for Biology, Indonesian Institute of Sciences (LIPI). Jl. Raya Jakarta- Bogor Km. 46, Cibinong, 16911, West Java, Indonesia.
[3]Department of Nutrition and Feed Tecnology, Bogor Agricultural University. Jl. Raya Darmaga, Bogor, 16680, West Java, Indonesia.
[4]Department of Biology, Bogor Agricultural University. Jl. Raya Darmaga, Bogor, 16680, West Java, Indonesia.
Email: nurjannah.suroso@gmail.com

Received 26 June 2015; Received in revised form 19 August 2015; Accepted 15 September 2015

ABSTRACT

Aims: An ecological study was conducted to investigate the diversity pattern of the lactic acid bacteria in the gastrointestinal tract of Cemani chicken, a native Indonesian chicken, using a molecular approach based on 16S rRNA genes.
Methodology and results: Digesta samples of seven chickens were collected for terminal restriction fragment length polymorphism (T-RFLP) analysis. The molecular diversity of lactic acid bacteria in crop, ventriculus, ileum and cecum were determined. The results showed that microbial composition of lactic acid bacteria in cecum was relatively different with other upper gastrointestinal tract. Lactic acid bacteria phylotypes and diversity in ileum were higher than those in the crop, ventriculus, and cecum.
Conclusion, significance and impact of study: We confirmed that cecum of native chicken has a different environment as compared to other gastrointestinal regions showing the lowest value of the Sorensen's index. This first report of LAB diversity pattern in Cemani chicken contributes a more comprehensive understanding of the microbial ecology in the chicken.

Keywords: Cemani chicken, gastrointestinal tract, lactic acid bacteria, microbial diversity, T-RFLP

INTRODUCTION

There are thirty one native chickens in Indonesia with various genotypic and phenotypic characteristics. The Cemani chicken is one of Indonesian native chicken showing unique morphological appearances with black colour concerns the whole body including nails, tongue, comb, bells, beak, feet, eye-balls, legs, feathers, skin, muscles, bones, and internal organs. This kind of chicken is mostly found in Kedu, Temanggung Regency, the central Java province (Nataamijaya, 2010). Commonly, the Cemani chicken in Indonesia is raised for eggs and meat production in the local market. The properties of eggs and meat in Cemani chicken have been intensively studied (Łukasiewicz *et al.*, 2009; Wahyuni *et al*., 2011). Sometimes, this chicken is also utilized in medicine as well as in ritual ceremonies (Sulandari *et al*., 2009).

Cemani chickens have been recognized to have high disease resistance, especially against viral disease and intestinal pathogens (Sulandari *et al.*, 2009; Nataamijaya, 2010). The ability of high disease resistance and adaptability to environmental conditions may be influenced by the balance of the intestinal microbiota, especially lactic acid bacteria (Lan *et al.*, 2004; Gaggia *et al.*, 2010). Lactic acid bacteria (LAB) are the most frequently applied chicken probiotic microorganisms (Patterson and Burkholder, 2003; Vargas-Rodriguez *et al.*, 2013; Jannah *et al*., 2014). Studies on the microbiota of broiler chickens have been carried out using both culture-dependent and culture-independent methods. Cultured base method showed microbiota density in the GI tract around 10^{10} - 10^{11} cells/g of digesta (Zhu *et al*., 2002; Walter, 2008). Lactobacilli population dominates in the crop because of its adherence ability (Fuller, 1975). Proventriculus has highly acidic conditions; therefore, the bacteria that survive are predominantly lactobacilli. The small intestinal region is dominated by *Lactobacillus* sp., *Enterococcus* sp., and *Escherichia coli*. Moreover, the

*Corresponding author

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

microbes found in the cecum are bacteroides, eubacteria, lactobacilli, bifidobacteria, and clostridia. However, only 10-60% cecum bacteria could be cultured (Barnes *et al.*, 1972; Salanitro *et al.*, 1974). Bacterial community analysis using culture-dependent methods have conducted, but these methods may suffer from biases, as only a small proportion of total bacteria count can be cultured (Zhu *et al.*, 2002; Zhou *et al.*, 2007). Recently, culture independent studies of lactic acid bacteria in the GI tract using molecular approach have been made, using the FISH method (Gerard *et al.*, 2008), DGGE method (Guan *et al.*, 2003; Walter *et al.*, 2008), or T-RFLP (Dunbar *et al*., 2000; Kaplan *et al.*, 2001; Lan *et al.*, 2004; Chen *et al.*, 2012).

A study LAB biodiversity in the GI tract of Cemani chicken in Indonesia has not yet been reported. Biodiversity study of LAB in Cemani chicken based on culture-independent method is important for more comprehensive understanding of the microbial ecology in the chicken. The aim of this study is to investigate the diversity pattern of the lactic acid bacteria in several regions of gastrointestinal tracts of Cemani chicken using the T-RFLP technique. Based on this basic scientific information, a strategy for developing domesticated poultry native chicken may be improved, especially in terms of the relation of the roles of microbiota on maintenance of healthy host condition.

MATERIALS AND METHODS

Bacterial strain

Strain *Lactobacillus salivarius* CSP004 isolated from cecum of Cemani chicken was used in this study. This strain is collection of Research Center for Biology, Indonesian Institute of Sciences. Culture was grown in MRS agar medium (De Man *et al.*, 1960) for maintenance and advanced studies.

Sample preparation

Total seven healthy Cemani chicken (7 to 12 month old; 1.0 to 1.5 kg body weight) were collected from Mranggen district, central Java, Indonesia. The chickens received no-antibiotic feed containing rice bran and leftover rice, and were maintained under a free-range system, so they consumed a diverse range of feed, including insects, worms, and various plant material. To analyse the microbial communities in chicken gastrointestinal tracts, animals were slaughtered and pooled digesta contents were collected from the crop, ventriculus, ileum, and cecum. The individual samples from chicken A and B were also collected for determining individual variation phylotypes. Gastrointestinal contents were then stored at –20 °C until further use.

DNA extraction

Amount of 0.1 g of bacterial culture colony and gastrointestinal contents of each sample were washed with 500 µL PBS buffer (phosphate buffer saline; pH 7.2) by centrifuging at 13,000 × *g* for 5 min (Zhu *et al.*, 2002) to remove impurities. Bacterial genomic DNA was extracted from pure cultures and from gastrointestinal contents using a Xprep Stool DNA Mini Kit (PhileKorea Technology, INC, Korea) per manufacturer instruction. DNA pellet was then resuspended in 50 µL TE buffer and stored at –20 °C.

PCR Amplification of 16S rDNA gene

A PCR mixture was prepared from each sample using a TaKaRa PCR Thermal Cycler Dice® Gradient (TAKARA BIO INC, Japan) with the 6-carboxyfluorescein-labeled primer 27F (5'-AGAGTT TGATCCTGGCTCAG-3') (Heilig *et al.*, 2002), which was synthesized by 1st BASE (Sequencing Genetika Science) and an unlabelled specific reverse primer for lactic acid bacteria S-G-Lab-0677 (5'-CACCG CTACACATGGAG-3') (Heilig *et al.*, 2002; Dicksved *et al*., 2007). PCR reaction mixture consisted of 25 µL GoTaq GreenMaster Mix (Promega, USA), 2 µL of each primer (10 pmol), and distilled water at a final volume of 50 µL and DNA template 100 ng in the final concentration. PCR condition was conducted with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation temperature at 94 °C for 30 sec, annealing temperature of 50 °C for 20 sec, and extension temperature at 68 °C for 40 sec, followed by a final elongation at a temperature of 68 °C for 7 min (Heilig *et al.*, 2002). PCR products were confirmed by electrophoresis of aliquots of PCR mixture (2 µL) in using 1% agarose gel in 1 × TAE buffer and visualized with ethidium bromide staining by using Gel Documentation System (ATTO Corporation, Japan). PCR products were purified using a GeneJet™ PCR Purification Kit (Thermo Fisher Scientific, USA) per manufacturer instructions. DNA obtained from the purification was added 40 µL nuclease free-water and stored at −20 °C until further uses.

Digestion of PCR products

The purified PCR products was digested with *Hae*III and *Msp*I restriction enzyme (Thermo Fisher Scientific, USA). Each reaction contains 15 units (1.5 µL) of restriction enzyme, 10× restriction buffer (2 µL), and 100 ng (1 µL) of sample DNA in 20 µL of final volume. Samples were incubated at 37 °C for 24 h, and inactivated with the temperature 95 °C according to the manufacturer's instructions. The restriction digest products were subjected to ethanol precipitation and dried.

T-RFLP analysis

Fragment analysis was conducted in the 1st Base Fragment Analysis Service (http://www.base-asia.com/fragment_analysis/). Aliquots (1 µL) of dissolved samples were then added to 10 µL of a mixture of formamide and a size standard (GeneScan-500 ROX; Applied Biosystems; Applied Biosystems) (100:5, vol/vol).

ISSN (print): 1823-8262, ISSN (online): 2231-7538

The fluorescently-labelled T-RFs were analysed by electrophoresis with an automatic sequence analyser (ABI PRISM 3100; Applied Biosystems) in gene scan mode, and the lengths of the T-RFs were determined by comparison with size standards using the Peak Scanner™ software v1.0 (Applied Biosystems).

Data analysis

The fluorescence signal can be distinguished from the noise by setting a threshold in which only the T-RFs are larger than or equal to 50 bp and percentage of peak area is larger than or equal 1% is used for further analysis (Li *et al.*, 2007). The relative peak areas of each T-RF were determined by dividing each raw area of the peak by the total area of peaks. The results that obtained in the decimal fractions form, rounded to the T-RF nearest, and was shown as a percentage value. A binary data table (presence or absence of T-RF) was generated, in which peaks with percentage of total area <1% were assigned as 0 (absence) and percentage of total area ≥1% was assigned as 1 (presence) (Chen *et al.*, 2012).

Diversity is evenness and richness of the bacterial community members detected as T-RFs by T-RFLP analysis (Dicksved *et al.*, 2007). Biodiversity value is determined based on species richness, total number phylotypes were found in sample (*S*) and Shannon-Wiener diversity index (H'). H' is calculated for each community as follows: $H' = -\sum p_i \ln (p_i)$ where p_i is the proportion of the total sample belonging to its phylotype. Sorensen's pairwise similarity index (C_s) was calculated for each pair of communities as follows: $C_s = 2j/(a+b)$, where j is the number of T-RFs of two profiles being compared, a and b are the number of T-RFs of each profiles, the index measure ranging 0-1, where a value 0 is no phylotype overlap between the communities, and a value 1 is exactly the same phylotypes are found in both communities; and number of specific phylotype. Every single T-RF is considered as one phylotype (sometimes refers to species) (Moeseneder *et al.*, 1999). To identify the phylotype, bacterial isolate *L. salivarius* CSP004 was used as an observed marker based on T-RF size. In addition, to identify other T-RFs, T-RFLP program analysis ISPAR (*in silico* PCR and Restriction) of MiCA (Microbial Community Analysis) III was performed based on RDP database (R10, U27) consisting of 1,519,356 bacterial 16S rRNA (http://mica.ibest.uidaho.edu/) (Shyu *et al.*, 2007).

RESULTS

This study describes the use of T-RF profiling for analysis of LAB biodiversity from several compartment in the gastrointestinal tract of Cemani chicken. The analysis of microbial community in complex ecosystems, such as gastrointestinal contents, requires a sufficient quality and quantity of DNA. Metagenomics DNA were obtained in this study and used as template for amplifying regions for determining microbial population of lactic acid bacteria by 16S rRNA gene amplification using the universal primer 27F and established primer specific for LAB, SG-Lab-0677-a-A-17. Successful amplification of 16S rRNA showed the fragment size about 650 bp (Figure 1). Furthermore, we proceed digestion of the amplified PCR product with *Hae*III and *Msp*I restriction enzymes for detecting 16S rRNA gene sequence polymorphisms. Fragment sizes obtained ranged from 53 to 584 bp. Since the internal standard used in this study was GS 500 ROX, probably whole member of lactic acid bacteria could not be observed completely. Based on MiCA3 online analyses that consisting of 1,519,356 bacterial 16S rRNA, we confirmed that most LAB had been covered (Table 1) and no unspecific target of LAB was observed in this study (http://mica.ibest.uidaho.edu/; Dicksved *et al.*, 2007).

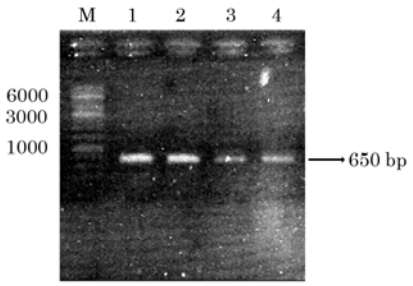

Figure 1: Amplified products of 16S rRNA gene recovered from the gastrointestinal tract of Cemani chicken using primer 27F-FAM and S-G-Lab 0677R: M, marker; 1, crop; 2, ventriculus; 3. ileum and 4. cecum.

For prediction, not all T-RFs could be classified to bacterial taxon using either *Hae*III or *Msp*I. Several of LAB phylotypes (41%) could not be identified refers to the available databases. The 63 and 163 bp T-RF peaks found in every community had not been identified as a known species by using MiCA3 - RDP databases. The existing database was limited, so this was a limitation of the T-RFLP method in this study in revealing the microbiota diversity. In addition, several T-RFs (17%) have been recognized as unculturable bacteria. In identification of phylotype, one T-RF sometimes could be determined as more than one species, such T-RF 244 bp could be expected as *L. amylovorus* or *L. helveticus*. Shared similar T-RF size (277 bp) was also found in *L. salivarius* or *L. delbrueckii.* These species were expected to be close relative strain in the taxonomy. The use of several restriction enzymes, sometimes, was applied to have clear identification of T-RFs in the fragment analysis for many intestinal bacteria.

Lactic acid bacteria community profiles of native chicken gastrointestinal tract

The differences in bacterial communities could be estimated by visual comparison of the electropherograms of T-RF patterns. Figure 2 shows a difference among microbial diversity in crop, ventriculus, ileum and cecum based on the *Hae*III profile. T-RF peaks of 63 and 163 bp

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 1: Microbial identification of T-RF peaks based on MiCA3 online database

Organism	T-RFs length (bp)	
	*Hae*III	*Msp*I
Lactobacillus		
L. salivarius	277	572
L. amylovorus	244	180
L. helveticus	244	180
L. crispatus	256	187
L. gasseri	322	189
L. rhamnosus	289	572
L. intermedius	323	NA*
L. delbruckii	277	180
L. curvatus	331	NA
L. acidophilus	244	180
L. lactis	331	572
L. brevis	NA	572
L. casei	NA	572
L. paracasei	NA	572
L. pentosus	NA	572
L. nantesis	NA	572
L. rennanqify	NA	572
Pediococcus		
P. pentosaceus	NA	584
P. cellicola	NA	584
Weissella		
Weissella cibaria	338	584
W. halotolerans	NA	584
W. confusa	338	NA
W. kandleri	338	NA
W. minor	338	NA
W. viridescens	338	NA

NA, not available in database

of *Hae*III profile always appear in every community. In the crop, 63 bp T-RF peak was recognized to be more abundant than the other communities. Our study demonstrated the gradual reduction of peak area relative abundance from the upper region to lower region of GI tract in Cemani chicken. Results also showed more abundant T-RF peak of 163 bp in cecum than those in other gastrointestinal regions.

In this study, strain CSP004 isolated from cecum one of native chicken was used as a marker of the presence representative LAB from intestine. This strain has been identified to close relationship with already known *L. salivarius,* based on 16S rDNA sequence analysis (data not shown). We observed the presence of 277 bp T-RF representing similarity to *L. salivarius* in ventriculus, ileum, and cecum. In addition, no significant peak of *L. salivarius* was detected in crop region (Figure 2).

The relative abundance of LAB phylotype in gastrointestinal tract could be understood from the presence of LAB phylotypes in the cecum. For examples, the 63, 163 and 277 bp T-RF were observed in cecum with different trend. The 92, 244 and 334 bp T-RFs observed in the crop, ileum and ventriculus communities were not found in the cecum community (Figure 3). We detected specific phylotypes with T-RF size that are found only in one community. Using *Hae*III profile, four (54, 78,116, 256 bp), one (289 bp), and three (158, 212, and 290 bp) specific T-RFs were identified in crop, ileum, and cecum, respectively. However, the specific phylotype of each community had low relative abundance (data not shown). Predominant phylotype were observed to be different in each community in which predominant phylotype in crop, ventriculus, ileum and cecum were T-RFs peaks of 244, 163, 334 and 163, respectively.

Diversity analysis of bacteria in the digestive tract

The number of LAB phylotypes in ileum (9.5) was higher than those of in other communities. The lowest number of phylotype was recognized in cecum (6.5) (Figure 4). Meanwhile, distinct member of phylotypes was observed by using different enzymes restriction. Most obtained data showed that the number of phylotypes by *Hae*III restriction enzyme produced more number of phylotypes than by using *Msp*I. As comparison, in the crop community, we found total 10 phylotypes by using *Hae*III restriction enzyme, whereas only 4 phylotypes were found using *Msp*I. In this study the average of data obtained from two restriction enzymes was used to estimate the species richness and diversity of LAB.

The Shannon-Wienner diversity index showed the diversity of LAB among four regions of gastrointestinal tract of Cemani chicken. Diversity index varied with the highest rank was determined in the ileum (2.13), whereas the lowest was in the cecum (1.08) (Figure 4). Sorensen's pairwise similarity index is a value which indicates the presence of two species in the community. A similarity value of 0 means that the communities are completely different from one another, while a value of 1 means that they are identical. In this study, the high similarity index of LAB composition (0.70) in the gastrointestinal tract in Cemani chicken between the ventriculus and ileum was observed (Table 2).

ISSN (print): 1823-8262, ISSN (online): 2231-7538

In Cemani chicken (composite sample of seven chickens), there were 17 phylotypes found at all of the community in the *Hae*III profile. Individually, there were differences in the number of phylotype found at local chicken. In chicken A and B, 18 and 24 phylotypes of LAB have been found, respectively. Seven phylotypes were found at individual A and B representing same phylotype as found in the composite sample (pooled sample). Six phylotypes were found in chicken A and B, but lacked in the pooled sample. Indeed, individual A and B had three and nine specific phylotypes, respectively. Altogether, these facts have demonstrated the phenomenon of inter-individual variation in the phylotypes richness among the samples (Figure 5).

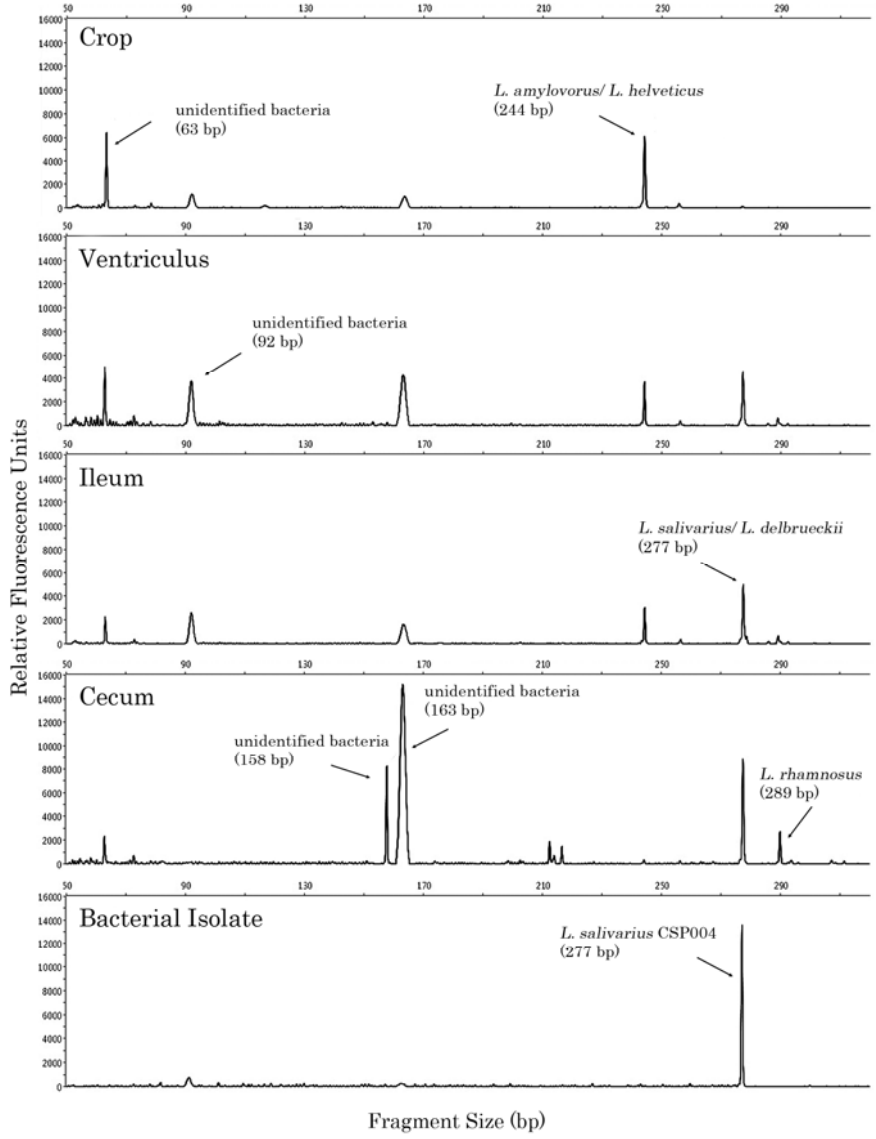

Figure 2: T-RFLP patterns of 16S rDNAs from gastrointestinal tract of Cemani chickens. Arrows indicate T-RF of LAB that identified based on MiCA3 database and reference bacterial isolate of *L. salivarius* CSP004 from chicken cecum.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

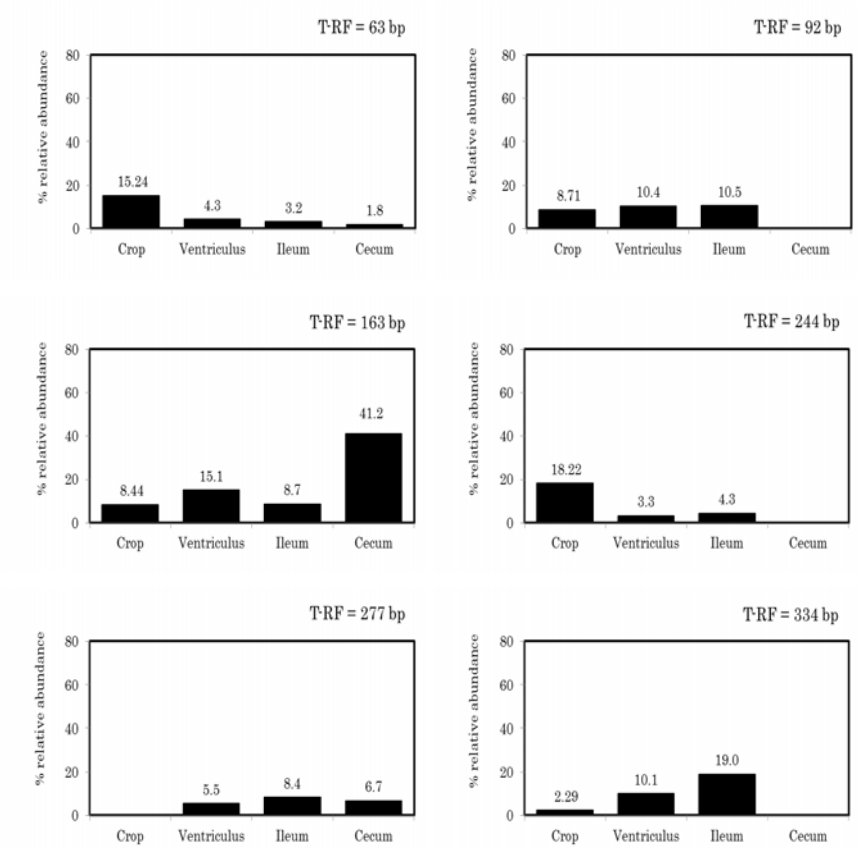

Figure 3: The relative abundance of LAB phylotypes in several parts of Cemani chicken gastrointestinal tracts. The number of T-RF base pair (bp) represents certain phylotype of LAB.

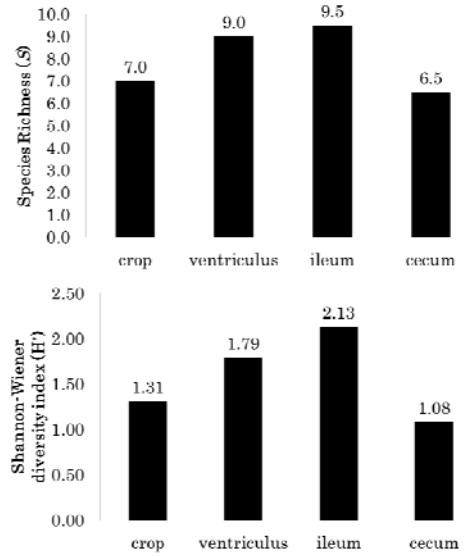

Figure 4: Diversity comparison of LAB in several parts of Cemani chicken gastrointestinal tracts. The numbers represent the diversity index of Shannon-Wiener.

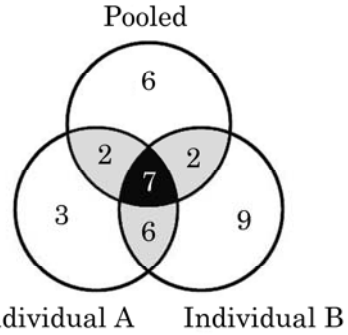

Figure 5: Total number of TR-Fs representing LAB phylotypes in the gastrointestinal tract of individual and pooled sample derived from seven chickens: common phylotype in all samples (black), shared phylotype only in two or several samples (grey), and phylotype found only in one sample (white).

DISCUSSION

Several changes in the gastrointestinal LAB community composition of Cemani chicken were observed in this study. In the crop, several phylotypes (63 and 244 bp T-

ISSN (print): 1823-8262, ISSN (online): 2231-7538

RF) were found more relatively abundant than the other region (Figure 2). These phylotypes may be responsible for the initial digestion of food entering gastrointestinal tract. Phylotype identified as *Lactobacillus amylovorus* or *L. helveticus* (244 bp T-RF) was gradually reduced during upper to lower parts and disappeared in cecum indicating the adaptation to environmental factors. *L. amylovorus* was reported as facultatively anaerobic, producing extracellular amylolytic enzyme, originally discovered from cattle waste-corn fermentations (Nakamura, 1981). *Lactobacillus. helveticus* identified in the same T-RF is a specialist dairy culture (Taverniti and Guglielmetti, 2012) which tolerates microaerophilic conditions. Interestingly, *L. crispatus* (256 and 187 T-RF peak of *Hae*III and *Msp*I profile, respectively) detected as a specific phylotype in crop region indicated poor adhesiveness to the other regions of the chicken intestinal tract or simply passing through the other regions of the chicken intestine. Our findings are in agreement with the previous study of abundant *L. crispatus* in the crop of chickens (Hilmi *et al*., 2007; Ojala *et al.,* 2010). *L. crispatus* adheres to the non-secreting stratified squamous epithelium of the chicken crop (Edelman *et al*., 2002). Most phylogroups in the chicken crop and gizzard microbiota were correlated to the *Lactobacillus*. Since ventriculus or gizzard play a role in mechanic digestion of food and influenced by the proventriculus acidic conditions, this region is not appropriate for growth of most bacteria. However, lactobacilli could tolerate environmental barriers in the ventriculus (Sekelja *et al*., 2012).

We observed the lowest similarity of LAB population in the community between the ileum and cecum regions. Higher similarity on microbial population in ventriculus and ileum than in other inter-regions reflected survivability of LAB against environmental barriers, such as enzymatic and acidic environment and the adherence ability to the intestinal tract. Our data for the Cemani chicken microbial community was similar to T-RFLP and 16S rRNA sequence analyses studies in broilers of a significant differences in bacterial population between the ileum and cecum (Gong *et al*., 2002). Based on 16S rRNA genes analysis, more than 70% of clones recovered from chicken ileal samples were related to lactobacilli and *Enterococcus cecorum* species. Whereas, *Faecalibacterium prausnitzii*, unidentified butyrate-producing bacteria, and uncultured bacteria were the predominant bacterial groups in cecum (Bjerrum *et al.,* 2006). The ileum has a higher diversity index than that of the other regions, indicating a more diverse LAB in the ileum. Presumably, this is related to convenient environment that supports a wide range of microbial growth. It was reported that several species derived from crop and successfully survived against the gastric barrier also help provide a variation of diversity in ileum (Hilmi *et al.,* 2007). LAB distribution in Cemani chicken as reveal by T-RFLP in this study was in consistent with the clone library-based study in conventional and organic broilers, in which most native gut microbiota in ileum belong to the *Lactobacillus* group (Bjerrum *et al.,* 2006).

In T-RFLP analysis, only dominant population was detected using this PCR-based approach (Blackwood *et al.,* 2007). The dominant phylotypes were successfully detected by PCR-based and T-RFLP analysis if they were belonging to predominant organisms (more than 1% of total microbiota). Therefore, rare species were difficult to be detected (Blackwood *et al*., 2007). In this study, number of LAB phylotype was lower in cecum than in other regions, although one phylotype detected was relatively high abundance (Figure 3). Since we only determine the LAB community in this study, the population of other microbial groups remain unknowns. However, a more diverse microbial community in cecum has been previously reported (Zhu *et al.,* 2002), and these are dominated by anaerobes (Gong *et al*., 2002; Lu *et al*., 2003).

It was estimated that only approximately 10% of bacteria in the broiler cecum were culturable. Genus *Lactobacillus* is one of major culturable cecal microbiota in chicken (24%), in which *L. salivarius* was recognized as predominant strain in this group (Lan *et al*., 2002). *Lactobacillus. salivarius* was reported as indigenous microbiota of the gastrointestinal tract in chicken with capability of probiotic properties (Nouri *et al*., 2010). The optimum conditions for cultivation performance of *L. salivarius was* studied by Lim *et al*., 2007. Our study demonstrated the presence of phylotype similar to *L. salivarius* in several regions of gastrointestinal tracts including ventriculus, ileum, and cecum. *L. salivarius* is facultative anaerobes that presumably adapted in the broad range of intestinal environment. The colonization of *L. salivarius* in the cecum was supported by several potential mechanisms including competition for nutrition by utilizing indigestible carbohydrates (Nilsson *et al*., 2006), competition for receptor site by producing exopolysaccharide (EPS) that helps to adhere to intestinal mucus (Stern *et al*., 2006; Raftis *et al*., 2011; Cao *et al*., 2012), and production of antimicrobial substances of salivaricin P (Barrett *et al*., 2007). A relatively high abundance of *L. salivarius* in the native chicken cecum presumably modulates the establishment the balance of microbiota in the cecum.

Undetected *L. salivarius* in the crop of Cemani chicken as demonstrated in this study was interesting, since this species was reported to be abundant in the crop to of broiler chickens (Guan *et al*., 2003; Hilmi *et al*., 2007). This phenomenon reflects the existence of host-modulated microbial phylotype. Recent study of pyrosequencing demonstrated that nutrients and the energy extracted from feed affect the microbial population in chicken intestine (Stanley *et al*., 2013). Thus, differences in the intensive farming and natural free-range systems have been suggested to influence the microbial community in chicken. In the intensive production system, broilers were maintained under controlled condition and received concentrated feed with supplementation of antibiotics and hormones (Bjerrum *et al.*, 2006). In contrast, as a native chicken, Cemani is raised under natural free-range system with low productivity and low nutrient input. Chicken in natural conditions enjoy a mixed

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

diets by scavenging from several sources including the household residues of kitchens, gardens, crop fields, orchards, harvest residues, and the environmental components of plant leaves and seeds, worms, insects, molluscs, stone, grit, and sand (Roberts and Senaratne, 1992; Sonaiya *et al*., 2002).

Although T-RFLP was not applied to quantify the total number of certain strain (Savichtcheva and Okabe, 2009) in the ecosystem, this molecular tool has been useful to characterize the community profiles and to roughly estimate the relative abundance of microbial phylotype based peak areas of digested amplified 16S rRNA genes. Several unidentified phylotypes found in other regions at relatively high abundance were not detected in the cecum (Figure 3). This is likely related to unsuitable environment of cecum for all bacteria. Cecum microbiota was dominated by anaerobes rather than lactobacilli which was mostly inhabited ileum (Lan *et al*., 2002). To identify the unidentified phylotypes in specific regions of gastrointestinal tract, further exploration of the native chicken microbiota using a combination of T-RFLP, clone library, and pyrosequencing is necessary.

CONCLUSION

We found that complexity in LAB diversity of Cemani chickens based on different T-RFs among the four communities, including crop, ventriculus, ileum, and cecum. The upper regions of gastrointestinal tracts (crop, ventriculus, and ileum) have similar in LAB composition to each other, but it was interestingly different when compared to lower region (cecum). Ileum has the highest phylotype richness and relative abundance, and also dominated by uncultured lactobacilli. Cecum has a different community than the others, and several specific phylotypes were found in each community. The diversity pattern of LAB in Cemani chicken in each region of gastrointestinal tract would be useful in terms of basic scientific information of microbial ecology in the chicken in order to develop domesticated poultry of native chicken. Thus, a strategy for modulating the growth and activity of autochtonous LAB in the chicken's gastrointestinal tract may improve the health condition of the host. In addition, LAB diversity pattern in Cemani chicken will offer more comprehensive understanding of the microbial ecology in the chicken.

ACKNOWLEDGMENTS

This work was supported by the collective grants from Diponegoro University and Research Center for Biology-Indonesian Institute of Sciences.

REFERENCES

Barnes, E. M., Mead, G. C., Barnum, D. A. and Harry, E. G. (1972). The intestinal flora of the chicken in period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. *British Poultry Science* **13, 311-326.**

Barrett, E., Hayes, M., O'Connor, P., Gardiner, G., Fitzgerald, G. F., Stanton, C., Ross, R. P. and Hill, C. (2007). Salivaricin P, one of a family of two-component antilisterial bacteriocins produced by intestinal isolates of *Lactobacillus salivarius*. *Applied and Environmental Microbiology* **73, 3719-3723.**

Bjerrum, L., Engberg, R. M., Leser, T. D., Jensen, B. B., Finster, K. and Pedersen, K. (2006). Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and cellular-based techniques. *Poultry Science* **85, 1151-1164.**

Blackwood, C. B., Hudleston, D., Zak, D. R. and Buyer, J. S. (2007). Interpreting ecological diversity indices applied to terminal restriction fragment length polymorphism data: Insights from simulated microbial communities. *Applied and Environmental Microbiology* **73, 5276-5283.**

Cao, L., Yang, X., Sun, F., Liu, C. and Yao, J. (2012). *Lactobacillus* strain with high adhesion stimulates intestinal mucin expression in broiler. *The Journal of Poultry Science* **49, 273-281.**

Chen, L., Teasdale, M. T., Kaczmarczyk, M. M., Freund, G. G. and Miller, M. J. (2012). Development of a *Lactobacillus* specific T-RFLP method to determine lactobacilli diversity in complex samples. *Journal of Microbiological Methods* **91, 262-268.**

De Man, J. C., Rogosa, M. and Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* **23, 130-135.**

Dicksved, J., Floistrup, H., Bergstrom, A., Rosenquist, M., Pershagen, G., Scheynius, A., Roos, S., Johan, S., Engstrand, L., Fahrlander, C. B. and Mutius, E. V. (2007). Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. *Applied and Environmental Microbiology* **73, 2284-2289.**

Dunbar, J., Ticknor, L. O. and Kuske, C. R. (2000). Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene Terminal Restriction Fragment Analysis. *Applied and Environmental Microbiology* **66, 2943-2950.**

Edelman, S., Westerlund-Wikstrom, B., Leskela, S., Kettunen, H., Rautonen, N., Apajalahti, J. and Korhonen, T. K. (2002). *In vitro* adhesion specificity of native lactobacilli within the avian intestinal tract. *Applied and Environmental Microbiology* **68, 5155-5159.**

Fuller, R. (1975). Nature of the determinant responsible for the adhesion of lactobacilli to chicken crop epithelial cells. *Journal of General Microbiology* **87, 245-250.**

Gaggia, F., Mattarelli, P. and Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. Review. *International Journal of Food Microbiology* **141, S15-S28.**

Gerard, P., Brezillon, C., Quere, F., Salmon, A. and Rabot, S. (2008). Characterization of cecal microbiota and response to an orally administered *Lactobacillus* probiotic strain in the broiler chicken. *Journal of*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Molecular Microbiology and Biotechnology **14, 115-122.**

Gong, J., Forster, R. J., Yu, H., Chambers, J. R., Wheatcroft, R., Sabour, P. M. and Chen, S. (2002). Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. *FEMS Microbiology Ecology* **41, 171-179.**

Guan, L. L., Hagen, K. E., Tannock, G. W., Korver, D. R., Fasenko, G. M. and Allison, G. E. (2003). Detection and identification of *Lactobacillus* species in crops of broiler of different ages by using PCR-denaturing gradient gel electrophoresis and amplified ribosomal DNA restriction analysis. *Applied and Environmental Microbiology* **69, 6750-6757.**

Heilig, H. G., Zoetendal, E. G., Vaughan, E. E., Marteau, P., Akkermansand, A. D. and de Vos, W. M. (2002). Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S Ribosomal DNA. *Applied and Environmental Microbiology* **68, 114-123.**

Hilmi, H. T. A., Surakka, A., Apajalahti, J. and Saris, P. E. J. (2007). Identification of the most abundant *Lactobacillus* spesies in the crop of 1- and 5-week-old broiler chicken. *Applied and Environmental Microbiology* **73, 7867-7873.**

Jannah, S. N., Dinoto, A., Wiryawan, K. G. and Rusmana, I. (2014). Characteristics of lactic acid bacteria isolated from gastrointestinal tract of Cemani chicken and their potential use as probiotics. *Media Peternakan* **37, 143-214.**

Lan, P. T. N., Hayashi, H., Sakamoto, M. and Benno, Y. (2002). Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. *Microbiology Immunology* **46, 371-382.**

Lan, P. T. N., Sakamoto, M. and Benno, Y. (2004). Effect of two probiotic *Lactobacillus* strains on jejunal and cecal microbiota of broiler chicken under acute heat stress condition as revealed by moleculer analysis of 16S rRNA genes. *Microbiology Immunology* **48,917-929.**

Li, F., Hullar, M. A. J. and Lampe, J. W. (2007). Optimization of terminal restriction fragment polymorphism (T-RFLP) analysis of human gut microbiota. *Journal of Microbiological Methods* **68, 303-311.**

Lim, C. H., Rahim, R. A., Ho, Y. W. and Arbakariya, B. A. (2007). Optimization of growth medium for efficient cultivation of *Lactobacillus salivarius* i 24 using response surface method. *Malaysian Journal of Microbiology* **3, 41-47.**

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J. and Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology* **69, 6816-6824.**

Łukasiewicz, M., Michalczuk, M., Pietrzak, D. and Adamczak, L. (2009). Chemical composition and physico-chemical properties of meat of Ayam cemani hens and cocks. *Animal Science* **46, 307-313.**

Kaplan, C. W., Astaire, J. C., Reddy, B. S., Sanders, M. E. and Kitts, C. L. (2001). 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology* **67, 1935-1939.**

Moeseneder, M. M., Arieta, J. M., Muyzer, G., Winter, C. and Herndl, G. J. (1999). Optimization of terminal restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* **65, 3518-3525.**

Nakamura, L. K. (1981). *Lactobacillus amylovorus*, a new starch hydrolyzing species from cattle waste-corn fermentations. *International Journal of Systemic Bacteriology* **31, 56-63.**

Nataamijaya, A. G. (2010). Pengembangan potensi ayam lokal untuk menunjang peningkatan kesejahteraan petani. *Jurnal Litbang Pertanian* **29, 131-138.**

Nilsson, U., Nyman, M., Ahrne, S., Sullivan, E. O. and Fitzgerald, G. (2006). *Bifidobacterium lactis* Bb-12 and *Lactobacillus salivarius* UCC500 modify carboxylic acid formation in the hindgut of ats given pectin, inulin, and lactitol. *The Journal of Nutrition* **136, 2175-2180.**

Nouri, M., Rahbarizadeh, F., Akhmadvand, D., Moosakhani, F., Sadeqzadeh, E., Lavasani, S. and Vishteh, V. K. (2010). Inhibitory effects of *Lactobacilus salivarius* and *Lactobacillus crispatus* isolated from chicken gastrointestinal tract on *Salmonella enteritidis* and *Escherichia coli* growth. *Iranian Journal of Biotechnology* **8, 32-37.**

Ojala, T., Kuparinen, V., Koskinen, J. P., Alatalo, E., Holm, L., Auvinen, P., Edelman, S., Westerlund-Wikstrom, B., Korhonen, T. K., Paulin, L. and Kankainen, M. (2010). Genome sequence of *Lactobacillus crispatus* ST1. *Journal of Bacteriology* **192, 3547-3548.**

Patterson, J. A. and Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poultry Science* **82, 627-631.**

Raftis, E. J., Salvetti, E., Torriani, S., Felis, G. E. and O'Toole, P. W. (2011). Genomic diversity of *Lactobacillus salivarius*. *Applied and Environmental Microbiology* **77, 954-965.**

Roberts, J. A. and Senaratne, R. (1992). The successful introduction of hybrid egg laying chickens into a Sri Lankan village. *Proceedings XIX World Poultry Congress*, Amsterdam **1, 818-821.**

Salanitro, J. P., Blake, I. G. and Muirhead, P. A. (1974). Studies on the cecal microflora of commercial broiler chicken. *Applied Microbiology* **28, 439-447.**

Savichtcheva, O. and Okabe, S. (2009). Qualitative and quantitative estimation of host-specific fecal pollution using *Bacteroides-Prevotella* 16S rRNA genetic

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

markers by T-RFLP and real-time PCR analyses. *Water Science Technology* **59, 1831-1840.**

Sekelja, M., Rud, I., Knutsen, S. H., Denstadli, V., Westereng, B., Næs, T. and Rudi, K. (2012). Abrupt temporal fluctuations in the chicken fecal microbiota are explained by its gastrointestinal origin. *Applied and Environmental Microbiology* **78, 2941-2948.**

Shyu, C., Soule, T., Bent, S. J., Foster, J. A. and Forney, L. J. (2007). MiCA: A web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA Genes. *Microbial Ecology* **53, 562-570.**

Sonaiya, E. B., Dazogbo, J. S. and Olukosi, O. A. (2002). Further assessment of scavenging feed resource base. *In:* Characteristics and Parameters of Family Poultry Production in Africa Results of a FAO/IAEA Coordinated Research Project. Dwinger, R. (ed.). IAEA, Vienna. **pp. 193-200.**

Stanley, D., Geier, M. S., Denman, S. E., Haring, V. R., Crowley, T. M., Hughes, R. J. and Moore, R. J. (2013). Identification of chicken intestinal microbiota correlated with the efficiency of energy extraction from feed. *Veterinary Microbiology* **164, 85-92.**

Stern, N. J., Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., Pokhilenko, V. D., Levchuk, V. P., Svetoch, O. E. and Seal, B. S. (2006). Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. *Antimicrobial Agents and Chemotherapy* **50, 3111-3116.**

Sulandari, S., Zein, M. S. A., Astuti, D. and Sartika, T. (2009). Genetic polymorphisms of the chicken antiviral Mx gene in a variety of Indonesian indigenous chicken breeds. *Jurnal Veteriner* **10, 50-56.**

Taverniti, V. and Guglielmetti, S. (2012). Health-promoting properties of *Lactobacillus helveticus*. *Front Microbiology* **3, 392-405.**

Vargas-Rodriguez, L. M., Duran-Melendez, L. A., Garcia-Masias, J. A., Arcos-Garcia, J. L., Joaquin-Torres, B. M. and Ruelas-Inzunza, M. G. (2013). Effect of probiotic and population density on the growth performance and carcass characteristics in broiler chickens. *International Journal of Poultry Science* **12, 390-395.**

Wahyuni, H. I., Suthama, N., Mangisah, I. and Sarjana, T. A. (2011). Egg quality and hatchability of *in situ* - reared Kedu and Cemani hens fed diet of farmer formulation supplemented with vitamin E. *Indonesian Journal of Tropical Animal and Agriculture* **36, 61-68.**

Walter, J. (2008). Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. *Applied and Environmental Microbiology* **74, 4985-4996.**

Zhou, H. J., Gong, J., Brisbin, J. T., Yu, H., Sanei, B., Sabour, P. and Sharif, S. (2007). Appropriate chicken sample size for identifying the composition of broiler intestinal microbiota affected by dietary antibiotics, using the polymerase chain reaction-Denaturing Gradient Gel Electrophoresis technique. *Poultry Science* **86, 2541-2549.**

Zhu, X. Y., Zhong, T., Pandya, Y. and Joerger, R. D. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chicken. *Applied and Environmental Microbiology* **68, 124-137.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538